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Circulating 25-hydroxyvitamin D, nasopharyngeal airway metabolome, and bronchiolitis severity

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Key Words: airway; bronchiolitis; metabolome; severity; vitamin D

ABSTRACT

Low circulating 25-hydroxyvitamin D (25OHD) levels are a risk factor for acute respiratory infection (e.g., bronchiolitis) in children. However, little is known about the relation of circulating 25OHD with the many downstream functional molecules in target organs – such as the airway – and with clinical outcomes. In this prospective multicenter study of infants (age <1 year) hospitalized with

bronchiolitis, we measured serum 25OHD levels and profiled the metabolome of 144 nasopharyngeal airway samples. Among 254 metabolites identified, we defined a set of 20 metabolites that are related to lower serum 25OHD and higher vitamin D-binding protein levels. Of these metabolites, 9 metabolites were associated with a significantly higher risk of positive pressure ventilation use. These metabolites were glycerophosphocholines esterified with proinflammatory fatty acids (palmitate, arachidonate, linoleate, and stearate), sphingomyelins, alpha-hydroxyisovalerate, 2-hydroxybutyrate, and 3-(4-hydroxyphenyl)lactate (all FDR<0.05). Based on the multicenter data, vitamin D-related airway metabolites were associated with risks of bronchiolitis severity.

BACKGROUND

Bronchiolitis is a common acute respiratory infection (ARI) and the leading cause of hospitalizations in U.S. infants.[1] Previous studies have investigated the role of vitamin D in bronchiolitis pathobiology because of its immune-modulating properties and likely role in lung development.[2] Indeed, 25-hydroxyvitamin D (25OHD) undergoes enzymatic conversion to its functionally-active form (1,25-dihydroxyvitamin D [1,25OH_2D]) in immune cells and epithelium cells.[2] Epidemiological studies have demonstrated associations of lower circulating 25OHD levels at birth or during infancy with higher risks of ARI, including bronchiolitis.[3-5] Additionally, recent individual participant data meta-analysis of 25 randomized controlled trials also showed protective effects of vitamin D supplementation against ARI among individuals with low baseline 25OHD levels.[6] Most of these studies have assessed the concentration of blood 25OHD directly in relation to disease processes.[3-7] However, little is known about relationships of circulating vitamin D compounds with the many downstream functional molecules in target organs – such as the airway – and with clinical outcomes. Metabolomics addresses this knowledge gap by systematically measuring all metabolites, which represent the downstream functional products of an individual's genetic make-up and environmental exposures, such as vitamin D status.[8]

In this context, by examining the nasopharyngeal airway metabolomic profiles from a nested subset of prospective cohort of infants with bronchiolitis, we aimed to examine the interrelationships between serum vitamin D-related compounds (25OHDs and vitamin D-binding protein [DBP]), airway metabolome, and disease severity. Specifically, we hypothesized that vitamin D-related airway metabolites are associated with higher risks of positive pressure ventilation (PPV) use.

METHODS

We analyzed the data from a multicenter prospective cohort study of infants hospitalized with bronchiolitis (severe bronchiolitis). Details of the study design, setting, participants, data measurement, and analysis may be found in the Online Supplement (**Supplemental Methods**). Briefly, this cohort study, the 35th Multicenter Airway Research Collaboration (MARC-35),[9,10] enrolled 1,016 infants (aged <12 months) hospitalized for bronchiolitis at 17 sites across 14 U.S. states (**Table E1**) during the 2011–2014 winter seasons. Bronchiolitis was defined by the American Academy of Pediatrics guidelines.[11] The institutional review board of each participating hospital approved the study. Written informed consent was obtained from the infant's parent or guardian.

In addition to the measurements of phenotypic data through structured interview and medical record review, we collected blood and nasopharyngeal airway samples within 24 hours of hospitalization using a standardized protocol.[9,10] Serum total 25OHD and DBP levels were quantified by immunoassays; serum free and bioavailable 25OHD levels were computed using previously-validated formulas.[12] Respiratory viruses were tested by using real-time PCR assays at Baylor College of Medicine (Houston, TX, USA) and nasopharyngeal metabolites (metabolome) were profiled using ultra-high performance liquid chromatography-tandem mass spectrometry by Metabolon (Durham, NC, USA). The details of these testing and their quality control may be found in **Supplemental Methods**. The

primary clinical outcome was the use of PPV – defined as the use of continuous positive airway pressure and/or intubation during hospitalization.[10]

For the current study, we analyzed 144 infants who underwent metabolomic profiling of nasopharyngeal airway samples. These 144 infants had sufficient amount of nasopharyngeal airway sample for metabolome testing. To identify a subset of nasopharyngeal metabolites related to lower levels of circulating 25OHD (free, bioavailable, and total), we used sparse partial least squares (sPLS) regression with cross-validation and LASSO penalization minimizing overfitting. Next, to examine the associations of these selected nasopharyngeal metabolites with risks of PPV, we fit multivariable logistic regression and generalized linear mixed-effects models adjusting for age, sex, race/ethnicity, feeding status, weight at presentation, detected virus, free 25OHD level, and potential patient clustering within hospitals. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate (FDR) method. Analyses used R version 3.4.

RESULTS

Of 144 infants with bronchiolitis in the analytic cohort, the median age was 3 months (IQR 1-6 months), 61% were male, and 17% underwent PPV during hospitalization. The median level of serum total 25OHD was 23.7 ng/ml (IQR 16.0-31.1 ng/ml) and that of free 25OHD was 10.0 pg/ml (IQR 7.5-15.0 pg/ml), with significant correlation between these levels (r=0.66; P<0.001; **Figure E1**). While most patient characteristics were not significantly different by free 25OHD status (**Table 1**), infants with lower free 25OH levels were more likely to be male, non-Hispanic white, and breastfed (all P<0.05).

Metabolomic profiling of nasopharyngeal airway samples detected a total of 254 metabolites from 62 sub-pathways within 8 super-pathways. Based on sPLS ordination, the global metabolomics profiles clustered by free 25OHD status (**Figure E2**). By estimating their loading coefficients in the sPLS model, we defined a set of top 20 nasopharyngeal metabolites that are most strongly associated with lower serum 25OHD (free, bioavailable, and total) levels, as well as higher DBP levels (**Figures**

1 and **E3**). These metabolites were products of lipid, amino acid, and carbohydrate metabolism. Of these nasopharyngeal metabolites, in the logistic regression models adjusting for potential confounders, 13 metabolites were significantly associated with a higher risk of PPV use (all FDR<0.05). In the mixed-effects models, 9 metabolites remained significantly associated with a higher risk of PPV use (all FDR<0.05) –glycerophosphocholines (GPCs) esterified with palmitate, arachidonate, linoleate, and stearate, sphingomyelins, alpha-hydroxyisovalerate, 2-hydroxybutyrate, and 3-(4-hydroxyphenyl)lactate (**Figure 1**). Additionally, the discrimination ability of selected 20 metabolites on the risk of PPV was high with area-under-the receiver operating characteristic curve of 0.95 (**Figure E4**).

DISCUSSION

In this multicenter study of infants with severe bronchiolitis, the levels of circulating vitamin D-related compounds were associated with differences in the nasopharyngeal airway metabolomic profile. Specifically, from a total of 254 detected metabolites, we derived a set of 20 airway metabolites – a metabolomic signature – that was associated with lower serum 25OHD (free, bioavailable, and total) levels. Of these, 9 airway metabolites (e.g., glycerophospholipids esterified with specific fatty acids, sphingolipids) were significantly associated with a higher risk of PPV use. To our knowledge, this is the first study that has investigated the interrelations between circulating vitamin D-related compounds, airway metabolome, and clinical outcomes in infants with bronchiolitis.

In agreement with our findings, studies of children[13,14] and adults[15-18] with various conditions (e.g., asthma, sepsis) have reported the relationship of lower 25OHD levels with unique blood and urine metabolomic profiles, particularly in lipids – a major component of cell membrane and molecular signaling.[8] For example, a nested study of 245 participants in a clinical trial reported that children exposed to lower *in utero* 25OHD levels had higher serum concentration of palmitate, stearate, linoleate, and arachidonate at age 3 years.[13] Cohort studies of adults in Germany and Finland

also demonstrated inverse associations between serum 25OHD levels and these fatty acids.[15,16] Palmitate and stearate are saturated fatty acids known to enhance inflammatory signaling.[13] Higher consumption of these saturated fats is linked to dyslipidemia, which is associated with allergic sensitization, airway obstruction, and childhood asthma.[19] Additionally, linoleate and arachidonate (ω-6 polyunsaturated fatty acids) are precursor of the arachidonic cascade that produces lipid mediators (e.g., leukotrienes, prostaglandins) and plays a major role in airway inflammation.[8] Furthermore, emerging evidence has also shown that $1,25OH_2D$ modulates metabolism of sphingolipids – signaling molecules involved in immune response and inflammation, which are related to airway diseases, including bronchiolitis[10] and asthma.[8]

In addition to these proinflammatory lipids, in agreement with our data, cross-sectional studies also reported associations of alpha-hydroxyisovalerate – a branched-chain amino acid – in urine with respiratory syncytial virus ARI in children[14] and asthma in adults.[17] Furthermore, a cross-sectional study of adult patients with sepsis also found relations of low 25OHD and high 2-hydroxybutyrate – an organic acid – levels in the serum with higher mortality.[18] Our study is consistent with these prior studies showing 25OHD-metabolome (in blood or urine) associations, and extends them by demonstrating the interrelations of circulating 25OHD, metabolome in the airway (the disease locus), and bronchiolitis severity in infants.

The current study has several potential limitations. First, the study was based on the nasopharyngeal samples that were measured <24 hours of hospitalization at a single time-point. Second, as with any observational study, the observed associations do not necessarily prove causality, and might be explained by reverse causation and unmeasured confounders (e.g., between-hospital difference in intensive care use). However, we statistically accounted for hospital-level effects. Third, it is possible that environmental exposures (e.g., medical management, diet) could have influenced the airway metabolome. Yet, given the long half-life of serum 25OHD and cell membrane lipids,[20] this factor was unlikely to have affected 25OHD status or confounded the observed associations of 25OHD with metabolome, particularly with cell membrane lipids. Fourth, our study did not include data from a "control" group. Regardless, the study objective was *not* to assess the relationship of

circulating 25OHD, airway metabolome, and the development of bronchiolitis (yes/no), *but* to determine their contributions to the disease severity among infants with bronchiolitis. Fifth, although we used statistical methods to minimize over-fitting, external validations in an independent population and mechanistic evaluations in experimental model systems are necessary to confirm our observations. Sixth, while an integration of other omics (e.g., genetics), environmental, and clinical data to systematically endotype bronchiolitis was out of the scope in the current study, it is an important area for examination in future work. Lastly, even with our multicenter design and racially/ethnically-diverse patient population, we must generalize our inferences cautiously beyond infants hospitalized with bronchiolitis.

In conclusion, these multicenter data of infants with bronchiolitis demonstrated that lower levels of serum vitamin D-related compounds were associated with a set of nasopharyngeal airway metabolites – a metabolomic signature. Of these, specific metabolites (e.g., glycerophospholipids esterified with proinflammatory fatty acids, sphingolipids) were significantly associated with a higher risk of PPV use. While causal inference remains premature, our data – in conjunction with prior studies – suggest that circulating 25OHD influences the airway metabolites related to inflammatory pathways, thereby contributing to disease severity. Our findings should facilitate further investigations into the complex interplay between the environmental exposures (e.g., vitamin D supplementation), host immune response in the airway, and bronchiolitis pathobiology.

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FIGURE LEGEND

Figure 1. Correlations between circulating vitamin D compounds and selected nasopharyngeal airway metabolites, and their associations with risks of positive pressure ventilation among infants with bronchiolitis

The heatmap (**left**) shows the correlations between the circulating vitamin D-related compounds and 20 selected nasopharyngeal metabolites that were examined using hierarchical clustering with average linkage algorithm. The table (**right**) summarizes the adjusted associations between each of selected nasopharyngeal metabolites with risks of PPV use. Bold results are statistically significant with FDR of <0.05. OR are calculated per each incremental increase in scaled level.

Abbreviations: 25OHD, 25-hydroxyvitamin D; CI, confidence interval; DBP, vitamin D-binding protein; FDR, false discovery rate; GPC, glycerophosphocholine; OR, odds ratio; sPLS, partial least squares.

* Logistic regression models adjusting for patients' age, sex, race/ethnicity, feeding status, body weight at presentation, detected virus, and serum free 25OHD levels.

† Generalized linear mixed-effects models adjusting for the covariates above and accounting for potential patient clustering within the hospitals.

Table 1. Patient Characteristics of Infants Hospitalized for Bronchiolitis by Serum Free 25-hydroxyvitamin D level

Variables	Free 25OHD <10 pg/ml n=71	Free 25OHD ≥10 pg/ml n=73	P-value
Characteristics			
Age (mo), median, (IQR)	2 (1-5)	3 (1-6)	0.11
Male sex	52 (73)	36 (49)	0.006
Race/ethnicity			0.006
Non-Hispanic white	39 (55)	31 (43)	
Non-Hispanic black	4 (6)	20 (27)	
Hispanic	23 (32)	18 (25)	
Other	5 (7)	4 (6)	
Parental history of asthma	27 (3)	18 (25)	0.12
Maternal smoking during pregnancy	6 (9)	6 (8)	0.99
C-section delivery	26 (37)	25 (35)	0.90
Prematurity (32-37 weeks)	13 (18)	18 (25)	0.47
Previous breathing problems before the index hospitalization*	5 (7)	12 (16)	0.14
History of eczema	8 (11)	9 (12)	0.99
Ever attended daycare	10 (14)	13 (18)	0.70
Sibling in the household			
Mostly breastfed for the first 3 months of age	45 (64)	27 (39)	0.004
Smoke exposure at home	5 (7)	11 (15)	0.21
Corticosteroid use before the index hospitalization	11 (16)	11 (15)	0.99
Clinical presentation			
Weight at presentation (kg), median (IQR)	5.3 (4.4-7.1)	6.3 (4.7-7.8)	0.23
Respiratory rate at presentation (per minute), median (IQR)	48 (39-60)	48 (40-59)	0.94

Oxygen saturation at presentation			0.81
<90%	8 (12)	7 (10)	
90%-93%	8 (12)	12 (17)	
≥94%	53 (77)	52 (73)	
Received corticosteroids (systemic or inhaled) during pre-hospitalization visit	5 (7.0)	6 (8)	0.99
Virology			
Sole RSV infection	43 (61)	37 (51)	0.31
Sole rhinovirus infection	12 (17)	14 (19)	0.89
RSV + rhinovirus coinfection	5 (7)	4 (6)	0.97
Others	11 (16)	18 (25)	0.25
Laboratory data			
Serum total 25OHD (ng/ml)†, median (IQR)	16.7 (8.9-23.7)	29.4 (23.1-34.4)	<0.001
Serum bioavailable25OHD (ng/ml), median (IQR)	2.4 (1.3-3.0)	4.9 (4.0-7.2)	<0.001
Serum vitamin D-binding protein (μg/ml), median (IQR)	180.0 (142.5-241.5)	132.9 (85.7-186.0)	<0.001
Serum LL-37 (ng/ml), median (IQR)	46.0 (28.0-60.0)	42.0 (30.0-58.0)	0.58
Food IgE sensitization‡	7 (10)	11 (15)	0.49
Aeroallergen IgE sensitization‡	1 (1)	3 (4)	0.63
Nasopharyngeal CCL5 (pg/ml), median (IQR)	38.0 (18.8-155.2)	53.9 (23.7-134.4)	0.75

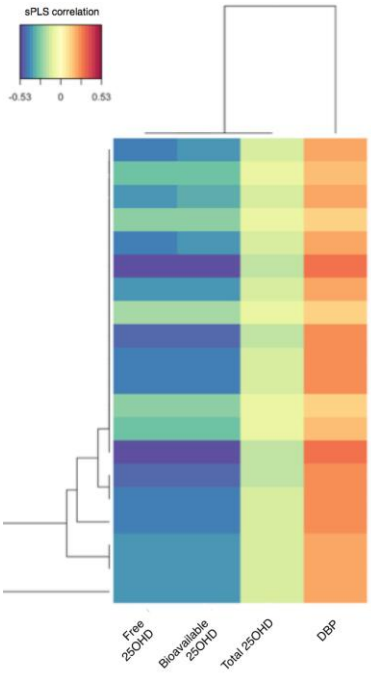
Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100, because of missingness

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; 25OHD, 25-hydroxyvitamin D

* Defined as a child having cough that wakes him/her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough

† Serum total 25OHD level was significantly associated with the risk of positive pressure ventilation (PPV) use (odds ratio per 1 ng/ml increase 0.94; 95%CI 0.89-0.98; P=0.005). In addition, younger age (P=0.01) and lower weight at presentation (P=0.007) were also associated with a higher risk of PPV use.

‡ Defined by having one or more positive values for allergen-specific IgE



Nasopharyngeal metabolites	Super pathway	Multivariable model 1*			Multivariable model 2†		
		OR (95% CI)	P-value	FDR	OR (95% CI)	P-value	FDR
1-palmitoyl-2-arachidonoyl GPC	Lipid	**1.24 (1.09-1.42)**	0.001	**0.006**	**1.38 (1.15-1.65)**	0.0005	**0.006**
1-linoleoyl-2-arachidonoyl GPC	Lipid	1.13 (1.00-1.27)	0.052	0.07	1.13 (0.98-1.31)	0.10	0.16
1-palmitoyl-2-linoleoyl GPC	Lipid	**1.39 (1.12-1.72)**	0.002	**0.008**	**1.55 (1.16-2.07)**	0.003	**0.01**
1-palmitoyl-2-palmitoleoyl GPC	Lipid	1.01 (0.99-1.04)	0.30	0.37	1.01 (0.98-1.04)	0.43	0.51
3-methyl-2-oxovalerate	Amino acid	1.07 (0.82-1.39)	0.62	0.62	1.00 (0.75-1.35)	0.98	0.98
alpha-hydroxyisovalerate	Amino acid	**1.89 (1.28-2.80)**	0.001	**0.006**	**2.16 (1.29-3.61)**	0.003	**0.01**
dimethylarginine	Amino acid	**1.38 (1.03-1.86)**	0.03	**0.049**	1.36 (0.95-1.97)	0.10	0.16
gluconate	Xenobiotics	**1.32 (1.03-1.68)**	0.03	**0.048**	1.27 (0.94-1.72)	0.12	0.17
glycochenodeoxycholate	Lipid	0.92 (0.72-1.17)	0.49	0.55	0.89 (0.67-1.19)	0.44	0.51
trans-4-hydroxyproline	Amino acid	**1.50 (1.06-2.12)**	0.02	**0.04**	1.42 (0.93-2.15)	0.10	0.16
kynurenine	Amino acid	**1.83 (1.11-2.99)**	0.02	**0.03**	1.96 (1.05-3.64)	0.03	0.07
ribulose-xylulose	Carbohydrate	1.18 (0.67-2.09)	0.56	0.59	1.04 (0.52-2.06)	0.92	0.96
sphingomyelin (d18:2/16:0;d18:1/16:1)	Lipid	**1.28 (1.07-1.54)**	0.008	**0.02**	**1.41 (1.10-1.80)**	0.006	**0.02**
taurochenodeoxycholate	Lipid	0.70 (0.44-1.10)	0.12	0.16	0.64 (0.37-1.11)	0.11	0.16
sphingomyelin (d18:1/18:1;d18:2/18:0)	Lipid	**1.47 (1.13-1.90)**	0.004	**0.01**	**1.79 (1.19-2.69)**	0.005	**0.02**
sphingomyelin (d18:1/20:1;d18:2/20:0)	Lipid	**1.57 (1.20-2.05)**	0.001	**0.006**	**1.82 (1.23-2.69)**	0.003	**0.01**
2-hydroxybutyrate	Amino acid	**2.06 (1.19-3.58)**	0.01	**0.02**	**2.45 (1.20-4.99)**	0.01	**0.03**
3-(4-hydroxypheny)lactate	Amino acid	**1.96 (1.31-2.95)**	0.001	**0.006**	**1.87 (1.18-2.97)**	0.008	**0.02**
citrulline	Amino acid	1.06 (0.94-1.21)	0.34	0.40	1.04 (0.86-1.24)	0.69	0.77
1-stearoyl-2-arachidonoyl GPC	Lipid	**1.31 (1.11-1.53)**	0.001	**0.006**	**1.50 (1.19-1.89)**	0.0006	**0.006**